Application Note

Quantifying lentiviral physical titer using the QIAcuity dPCR system

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Abstract

Developing safe and effective cell and gene therapies is essential for treating various diseases. A key component of these therapies is the use of lentiviral vectors (LV), a robust technology for delivering therapeutic genes into cells. To help ensure their safety and efficacy, lentiviral preparations must undergo rigorous quality control, including accurate quantification of viral vector genome titers and vector copy number (VCN) and impurity detection. Digital PCR (dPCR) offers precise, accurate and reproducible quantification, addressing the limitations of qPCR. We developed a direct reverse transcription digital PCR (RT-dPCR) approach for LV titer estimation that does not require RNA extraction or purification and demonstrates comparable performance to RT-dPCR using purified RNA. Our streamlined dPCR workflow enhances LV characterization during biopharmaceutical manufacturing, making it ideal for process development, lot release testing and optimization of viral doses in therapeutic applications.

Introduction

Lentiviruses belong to the Retroviridae family. Many of these viruses cause slowly progressive, chronically degenerative diseases, while others are nonpathogenic. Lentiviruses are enveloped particles with an approximate diameter of 80-100 nm. They are spherical and contain two copies of positive-sense single-stranded RNA bound by nucleocapsid proteins. The particles also contain reverse transcriptase, integrase and protease proteins. Lentiviruses enter cells through an interaction between glycoproteins on their outer envelope and specific cell receptors. Upon entry, lentiviral DNA integrates into the host genome in a non-random manner, preferring transcriptionally active sites.

Lentiviral vectors are ideal for therapeutic transgene delivery due to their ability to integrate into the host genome, enabling long-term expression. They can package up to 9 kb and express multiple genes from a single vector, which is crucial for treating complex diseases. Unlike gammaretroviral vectors, lentiviral vectors can transduce non-dividing cells and elicit only weak immune responses, making them more efficient for therapeutic applications.

Lentiviruses play a pivotal role in cell and gene therapy. They enable innovative treatments for genetic disorders, enhance cancer immunotherapy through CAR-T cells and support the development of effective vaccines for infectious diseases. They are also widely used to deliver CRISPR-Cas systems, enabling precise gene editing in various cell types. Increasingly, lentiviral vectors are applied in vivo gene therapy, where the therapy is delivered directly to the patient's cells, reducing the need for complex cell manipulation. Viral titer assessment is a critical step in lentivirus manufacturing. It supports process optimization and ensures accurate dosing for cell transduction at the desired multiplicity of infection (MOI), contributing to the development of safe, effective cell and gene therapies. ▷

Commonly used methods for non-functional and functional lentiviral titer quantitation include p24 ELISA, RNA vector genome titers by reverse transcriptase (RT)-qPCR or digital PCR, reverse transcriptase activity (RT) and fluorescence-activated cell sorting (FACS) flow cytometry.

There is an increasing need for fast and robust quantitation methods for lentiviruses in cell and gene therapy (CGT). We have developed the new CGT Lentivirus Lysis Kit and protocol, which, combined with QIAcuity CGT dPCR assays, enables vector genome titer quantification with fewer hands-on steps in a direct one-step reverse transcription digital PCR approach without requiring RNA extraction and purification.

Direct digital-PCR-based RNA detection of lentiviral genomes offers significant advantages. This method is very fast and straightforward, reducing delays between harvest and target cell infection, allowing both processes to occur on the same day. This is particularly beneficial for the time-sensitive manufacturing of CAR-T cells. It also helps to avoid freeze-thaw cycles, which have been shown to reduce the infectivity of lentiviral vectors. Using lysate directly as input eliminates the need for RNA isolation, reducing hands-on steps, sample-to-sample variability and loss of material during sample preparation.

Furthermore, dPCR improves qPCR by eliminating tedious and error-prone standard curve dilution and pipetting, and by providing accurate absolute quantification.

Results

One-step RT-dPCR method for direct lentiviral particle quantitation

We developed a streamlined method for direct lentiviral particle quantitation using one-step RT-dPCR without the need for viral RNA isolation. Recombinant lentivirus particles are commonly produced through co-transfection of several plasmids into a packaging cell line, usually HEK293T. Hence, LV preparations often contain significant amounts of DNA contaminants. Therefore, the first step of our approach comprises DNase I treatment to remove residual plasmid and host cell DNA. Afterward, the LV samples are lysed in CGT Dilution Buffer in the presence of Proteinase K, followed directly by quantitation of the viral genomes in a one-step RT-dPCR reaction using the OneStep Advanced Probe Master Mix on the QIAcuity dPCR instrument (Figure 1).

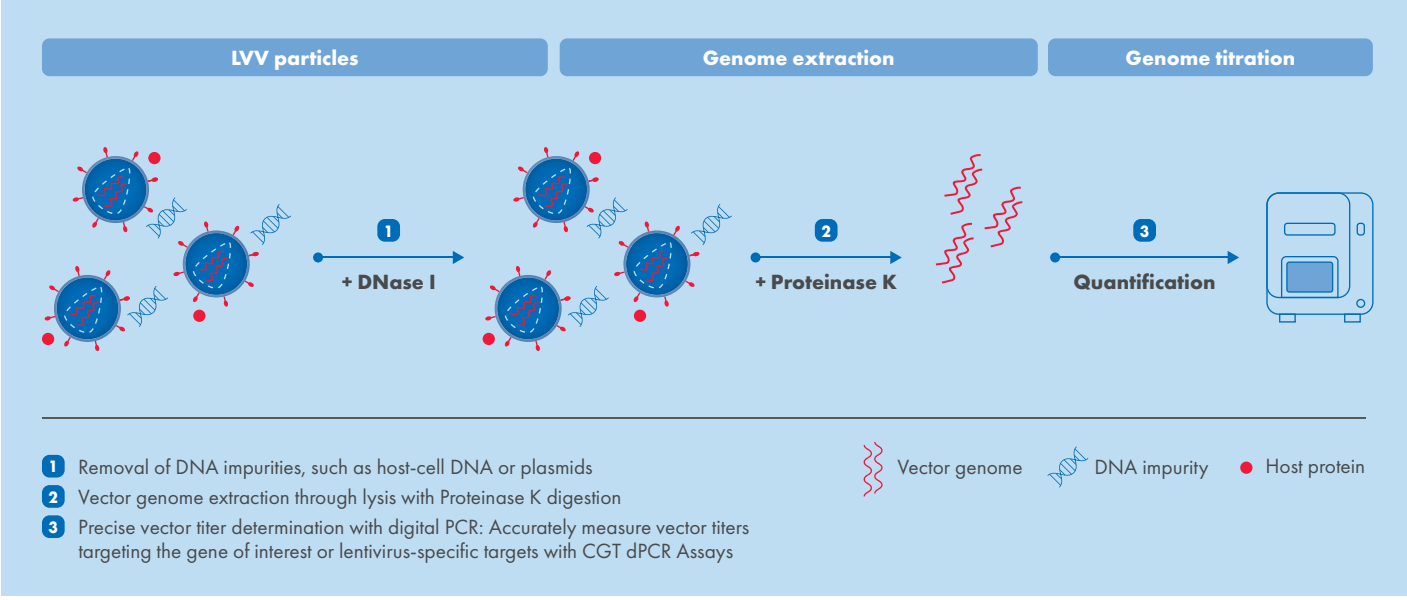

Figure 1. LV sample processing and titer determination using QIAGEN CGT Lentivirus Lysis Kit and CGT dPCR Assays: A schematic overview. In the first step, LV samples are treated with DNase I to remove DNA impurities such as residual plasmid DNA or host cell DNA. In step two, DNase I-treated samples are lysed in the presence of Proteinase K. Finally, in step three, the viral titers are determined with lentivirus-specific dPCR CGT Assays in combination with the OneStep Advanced Probe Master Mix using a QIAcuity dPCR instrument.

LV titer calculation from dPCR data

The raw values obtained with our CGT lentivirus dPCR quantitation workflow are in the format of copies/µL. To convert the dPCR readout into a more common unit for LV physical titers, namely viral particles per milliliter (VP/mL), the following calculation is performed:

LV titer (VP/mL) = [copies/µL x total dilution during CGT Lentivirus Lysis procedure x (dPCR reaction volume per well ÷ sample input per well) x 1000] ÷ 2*

*Each virion contains two single-stranded RNA genomes; therefore, the obtained dPCR readout is divided by two.

In-process samples are compatible with the CGT Lentivirus Lysis Kit

To evaluate the compatibility of our newly developed direct LV quantitation method with in-process samples from cell-based bioproduction systems, which typically contain a range of impurities that could inhibit precise quantitation, we processed crude lentivirus-containing cell culture supernatants using both the CGT Lentivirus Lysis Kit and the QIAamp RNA Viral Mini Kit in parallel. We then compared the titers obtained from each approach using our LV-specific dPCR CGT Assays for 5'-LTR, Psi and RRE in multiplex. Interestingly, the titers measured using the direct RT-dPCR technique with the CGT Lentivirus Lysis Kit were higher than those determined for supernatant samples where viral RNA had been purified prior to RT-dPCR (Figure 2).

High-titer lentiviral samples can be processed with the CGT Lentivirus Lysis Kit

Since lentiviral samples are typically concentrated after harvest during the production process, we evaluated the suitability of our direct CGT Lentivirus quantitation workflow for use with highly concentrated crude samples in complete DMEM and PBS. Freshly harvested LV supernatant samples were concentrated approximately 100-fold by PEG precipitation and resuspended either in complete DMEM or PBS. The resulting concentrates were subjected to either the new CGT Lentivirus Lysis Kit workflow or processed with the QIAamp RNA Viral Mini Kit, and the physical titer was subsequently determined in a multiplex dPCR setup using the LV-specific dPCR CGT Assays (5'-LTR, Psi and RRE) and the OneStep Advanced Probe Master Mix on a QIAcuity dPCR instrument. As shown in Figure 3, the lysates from our recently developed CGT Lentivirus Lysis Kit gave comparable results for the DMEM and the PBS sample, exceeding the corresponding titers obtained for the extracted and purified samples. These results were consistent with our earlier findings using in-process samples (Figure 2), where higher yields were also observed with the CGT Lentivirus Lysis Kit.

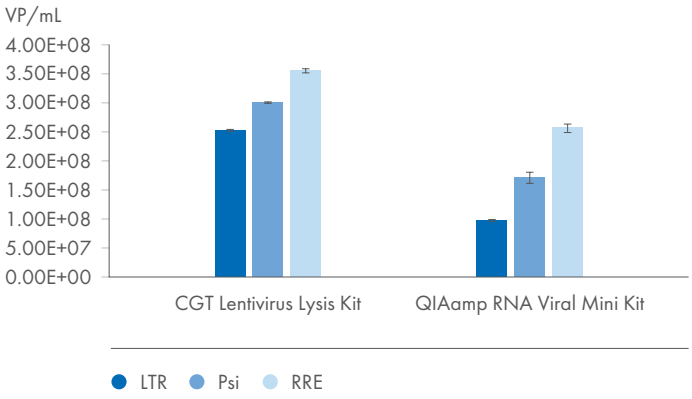

Figure 2. Comparison of titers from crude LV cell culture supernatant determined with one-step RT-dPCR. For titer determination, cell culture supernatant from the harvest step during lentivirus production was either processed with a direct RT-dPCR approach using the CGT Lentivirus Lysis Kit or with RNA extraction and purification using the QIAamp RNA Viral Mini Kit prior to one-step RT-dPCR measurement.

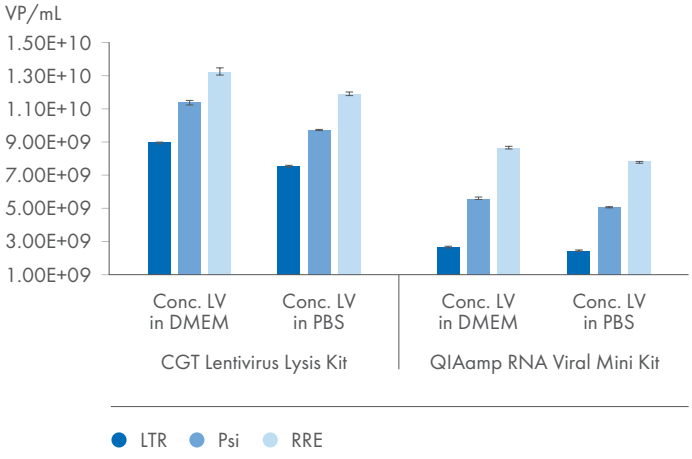

Figure 3. Concentrated LV samples can be processed with the CGT Lentivirus Lysis Kit. PEG-precipitated lentiviral samples in complete DMEM or in PBS were processed with either the CGT Lentivirus Lysis Kit or the QIAamp RNA Viral Mini Kit. Physical titers were then quantified using one-step RT-dPCR in multiplex with three dPCR CGT Assays specific for LV targets (5'-LTR, Psi and RRE).

Consistent total viral particle quantitation across various lysate amounts

Samples obtained at various stages of the lentivirus production process may exhibit a wide range of viral particle titers. Therefore, flexibility in direct lysate input volume is advantageous for achieving a broad detection range in one-step RT-dPCR. To test this, we lysed an LV supernatant sample with the CGT Lentivirus Lysis Kit and assessed viral titers using input volumes ranging from 1.2 µL (10% of total RT-dPCR reaction) to 6 µL (50% of total RT-dPCR reaction). The dPCR results showed that viral particle quantitation remained stable across varying lysate input amounts, indicating that our newly established direct lentivirus quantitation method utilizing one-step RT-dPCR is a robust and reproducible method for physical titer determination of lentiviral samples (Figure 4).

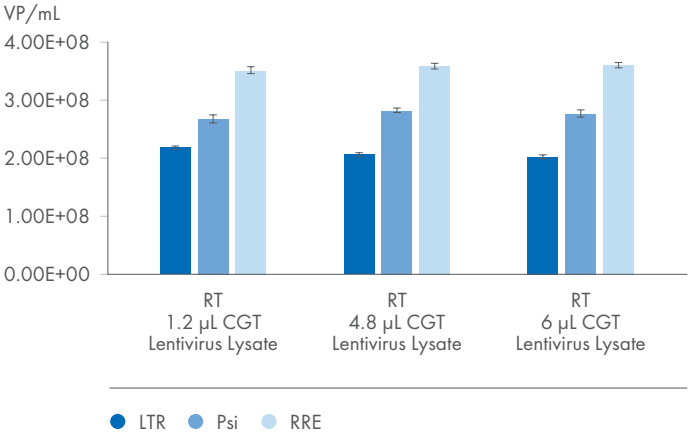

Figure 4. Consistent viral particle quantitation over varying lysate amounts. An LV-containing cell culture supernatant was processed with the CGT Lentivirus Lysis Kit, and a range of lysate volumes was used for quantitation via one-step RT-dPCR. Three CGT dPCR Assays specific to LV targets (5'-LTR, Psi and RRE) were used in multiplex.

Stable quantification of lentiviral titers following extended incubation in dPCR nanoplates

During the development and optimization of new LV production processes, a large number of samples may accumulate that require characterization, creating potential delays in analysis. To assess the robustness of our method, we tested whether lentiviral quantitation remains stable after prolonged incubation. We processed various sample types using our newly established LV quantitation workflow and measured the lysates immediately on the QIAcuity dPCR System or after a 14-hour room temperature incubation in nanoplates. As shown in Figure 5, the total LV particle quantitation values obtained after the one-step RT-dPCR reactions with LV lysates incubated for an extended period were found to be highly comparable to the samples immediately quantified. These results confirmed the robustness and stability of our workflow even under high-throughput conditions.

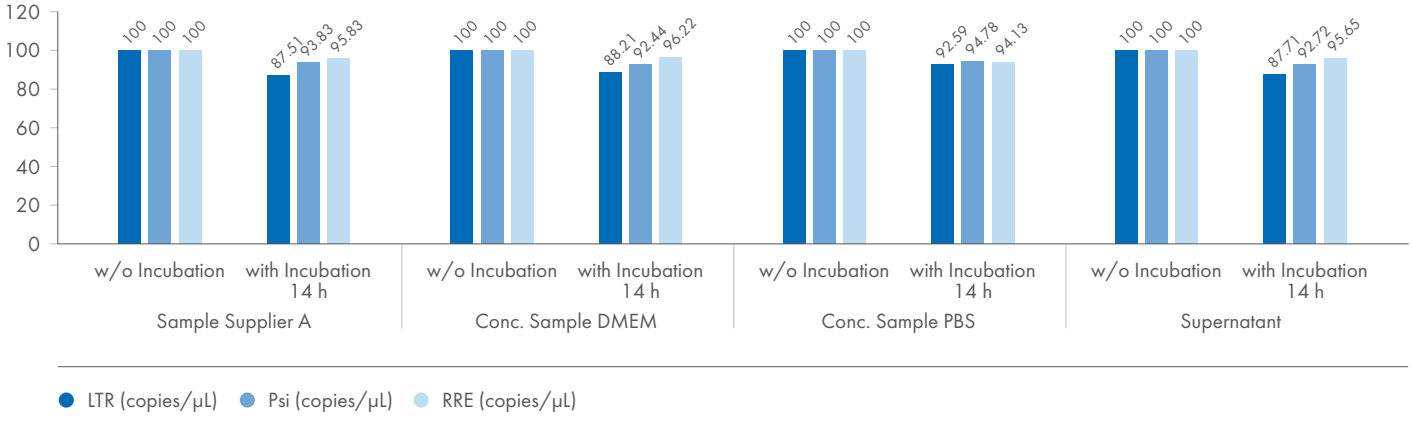

Figure 5. Quantitation of total LV particles remains stable after a 14-hour incubation in dPCR nanoplates. Four different types of LV samples were processed using the CGT Lentivirus Lysis Kit. The corresponding lysates were either measured immediately via RT-dPCR (multiplexed with three CGT dPCR Assays specific for LV targets: 5'-LTR, Psi and RRE) or incubated in nanoplates for 14 hours prior to measurement. The data shown are % of quantitation compared to immediate measurement.

Correlation of physical titer to functional titer values

Determination of the functional titer (TU/mL or IFU/mL) of recombinant lentivirus stocks, while being the more relevant measurement for downstream applications, usually involves (re)infection of cells and a subsequent readout method (e.g. FACS or quantification of integration events), making it both time-consuming and laborious. In contrast, our recently developed direct lentiviral particle quantitation approach using one-step RT-dPCR technology allows rapid and streamlined total viral particle quantitation within only a few hours and with minimal hands-on steps. However, it provides only the physical titer (VP/mL), which is typically 10-100-fold higher than the functional titer.

For example, functional titer determination can be performed by measuring the number of integrated lentiviruses in the genome of the transduced cell line using one or more of our lentivirus-specific QIAcuity Cell and Gene Therapy (CGT) dPCR Assays in combination with one of our three CGT dPCR Assays targeting a human reference gene (Albumin, RPL32 or RPP30), providing an accurate measure of the number of infectious viral particles capable of integrating into the host genome. It is possible to establish a correlation between these two values, the physical and the functional titer, for viral samples produced using the same packaging method and process. This is achieved by performing both types of measurements for the sample in parallel and then calculating an infectivity coefficient by dividing the physical titer value by the functional titer value: (VP/mL) ÷ (IFU/mL).

The resulting coefficient can later be used to estimate the infectivity of other lentiviral stocks produced using the same method by applying it to the physical titer value determined using the CGT Lentivirus Lysis Workflow. This approach significantly reduces time delays between LV harvest and target cell transduction and helps avoid the adverse effects of freeze-thaw cycles on viral stocks.

Summary

The physical lentiviral titer is one of the critical quality attributes during the biomanufacturing process of lentivirus-based therapeutic approaches. Accurate and rapid quantitation of the essential titer is vital throughout the optimization of a lentivirus production process, for in-process quality control during manufacturing, lot release testing and optimizing viral doses. Consequently, we developed a straightforward method for total lentiviral particle quantitation employing one-step RT-dPCR technology.

Our approach does not require RNA isolation, saving time and reducing handling steps and variability. Since our workflow includes a DNase I step (Figure 1), inaccuracies caused by residual plasmid DNA or host cell DNA are minimized during physical titer determination on the QIAcuity dPCR System. Furthermore, our approach utilizes one-step RT-dPCR chemistry, which makes it more sensitive and precise than other procedures. We demonstrated that our direct workflow is compatible with both crude in-process samples (Figure 2) and highly concentrated lentiviral samples (Figure 3), and that it can therefore be used at any step during the entire manufacturing process. A wide range of lentiviral particle titers can be processed using our system. Lysate input amounts can be varied without loss of measurement accuracy (Figure 4), making it an optimal method for quantitation at various production steps.

During periods of high sample throughput, delays between processing and measurement of samples can occur. We showed that prolonged incubation times of lysates in the reaction mix do not adversely affect measurement outcomes (Figure 5). To minimize delays between LV production and target cell infection, it is also feasible to establish a correlation coefficient for converting the physical titer determined with our direct CGT Lentivirus Lysis workflow to a functional titer, as long as the LV production process remains constant.

Conclusion

The CGT Lentivirus Lysis Kit provides a rapid and robust solution for determining lentivirus titers. This kit simplifies the dPCR process for measuring total RNA lentiviral genomes on the QIAcuity dPCR System, resulting in enhanced precision, accuracy and reliability.
Key benefits include optimized LV titer assessment through a mix-and-match approach using assays designed for lentiviral elements such as 5´LTR, Psi or RRE or regulatory elements like WPRE or the CMV promoter/enhancer. Additional assays targeting the gene of interest can be included in a multiplex approach. The workflow increases efficiency and reliability by eliminating the need for RNA purification and standard curves, reducing hands-on time, turnaround time (TAT) and errors, while enhancing accuracy and consistency. All assays are standardized and wet-lab tested, ensuring consistent performance. Overall, the CGT Lentivirus Lysis Kit streamlines lentivirus titer determination, offering a reliable and efficient solution for researchers, developers and QC labs.

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